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Challenges to targeting epidermal growth factor receptor in glioblastoma: escape mechanisms and combinatorial treatment strategies

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Abstract

Epidermal growth factor receptor (EGFR) gene amplification as well as activating mutations are common findings in glioblastomas. EGFR is on top of a down-stream signalling cascade which regulates important characteristics of glioblastoma cells including cellular proliferation, migration and survival. Targeting EGFR has therefore been regarded a promising therapeutic strategy in glioblastoma for decades. However, although various pharmacological inhibitors and anti-EGFR antibodies are available, the anti-glioma activity of these agents has been largely limited to preclinical models whereas their administration to glioblastoma patients was characterized by lack of clinical benefit. Comprehensive efforts have been made within the last years to understand the underlying mechanisms which confer resistance to EGFR inhibition in glioma cells. The absence of well-known mutations which predict response to EGFR tyrosine kinase inhibitors in gliomas as well as the presence of redundant and alternative compensatory pathways are among the most important escape mechanisms that prevent potent anti-glioma effects of EGFR-targeting drugs. Accordingly, an increasing number of *in vitro* and *in vivo* studies aimed at overcoming this resistance by combinatorial approaches using anti-EGFR treatment together with one or more additional drugs. Novel insights into the molecular mechanisms mediating resistance to anti-EGFR treatment and promising combinatorial approaches may help to better define a future role for EGFR inhibition in the treatment of glioblastoma.

Background

Gliomas are the most common primary brain tumors in adults. They are classified according to the World Health Organisation (WHO) into grades I-IV with glioblastoma being the most malignant subtype. Despite all efforts, median survival in glioblastoma patients is restricted to approximately 16 months in clinical trial populations ¹. Various therapeutic strategies have been explored within the last years in order to improve the prognosis of glioblastoma patients. Several of these novel strategies aim at targeting specific molecules or signalling pathways that are deregulated in glioma cells. Among the genetic aberration associated with gliomas, amplification of the epidermal growth factor receptor (EGFR, also named HER1 or ERBB1) is a frequent finding which has been described in approximately 40-50% of all glioblastomas ². Besides EGFR, the family of HER receptor tyrosine kinases comprises ERBB2 (more frequently known as HER2/neu), ERBB3, and ERBB4. EGFR binds several ligands including epidermal growth factor (EGF), transforming growth factor (TGF)- α , heparin-binding EGF-like growth factor (HB-EGF), amphiregulin, betacellulin, epigen and epiregulin ³. Engagement of EGFR results in the activation of a cytoplasmatic tyrosine kinase (TK) domain and subsequent intracellular downstream signalling involving, among others, the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) pathways ². Thus, EGFR signalling affects various cellular processes including proliferation, survival and metabolism. Amplification of EGFR is frequently associated with the occurrence of a mutant form of EGFR called EGFR variant III (EGFRvIII, also known as Δ EGFR). EGFRvIII is found in approximately 25-30% of all glioblastomas. However, there are various other mutations in EGFR, some of which predict a response to pharmacological inhibitors (see below). Because of its role as a central regulator of various biological processes in glioma cells as well as its potential contribution to resistance to apoptotic stimuli

and alkylating chemotherapy with temozolomide ^{4,5}, EGFR has attracted much attention as a therapeutic target.

Resistance to pharmacological EGFR inhibitors and antibodies targeting EGFR

As outlined above, EGFR has been regarded as a promising point of attack for therapeutic interventions against malignant gliomas. However, most approaches used so far have shown disappointing results in the clinic with virtually no benefit for populations of unselected patients. Thus, a major research focus within the last years has been the deciphering of the molecular mechanisms underlying the resistance of glioma cells to EGFR inhibition. The following section describes EGFR-targeted therapies as well as molecular alterations that may confer resistance to EGFR inhibition.

Pharmacological EGFR inhibitors

Pharmacological inhibitors, mostly small molecule tyrosine kinase inhibitors (TKI), targeting EGFR have been extensively tested in preclinical glioma models. Similar to other tumor entities such as lung carcinomas, where these drugs are well established in clinical practice, most investigators used erlotinib or gefitinib to interfere with EGFR signalling. The EGFR-blocking activity of erlotinib and gefitinib largely depends on the presence of mutations in exons 19 and 21 of the TK domain. These mutations are commonly found in lung cancer and other tumor entities and led to the approval of several EGFR inhibitors. However, these “sensitizing” mutations are virtually absent in glioblastomas, which may partially explain the lacking activity of standard TKI in this disease ⁶⁻⁹.

Antibodies against EGFR

Antibodies directed against EGFR with cetuximab, nimotuzumab and panitumumab as the most prominent candidates were also investigated for their anti-glioma activity *in vitro* and *in vivo*. Antibodies may exert their effect by preventing the binding of EGFR ligands to the receptor. Furthermore, antibody binding may result in receptor internalization and degradation¹⁰. Although antibodies to EGFR have been approved for other cancer types, e.g., cetuximab for the treatment of KRAS wild type colon cancer, their use against intracranial neoplasms such as glioblastoma represents a challenge due to the presence of the blood-brain barrier which may preclude the penetration of the antibody to all parts of the tumor. However, small molecule EGFR inhibitors such as erlotinib and gefitinib did also not markedly inhibit EGFR phosphorylation *in vivo*¹¹. Accordingly, the blood-brain barrier may represent an important “resistance factor” that limits the activity of EGFR-targeting drugs in the brain.

General mechanisms of resistance to EGFR-targeted therapies

The escape of glioma cells from EGFR-targeted therapy is caused by several characteristics of these cells and particularly by the existence of multiple overlapping and alternative compensatory signalling pathways which allow for a loss of EGFR function without detrimental effects on the cells^{12,13}. One common finding in glioma cells, that is, loss of phosphatase and tensin homolog deleted on chromosome 10 (PTEN) has been identified as a resistance factor to drugs directed against EGFR. PTEN loss promotes resistance to EGFR presumably by dissociating EGFR inhibition from down-stream inhibition of the PI3K pathway^{7,14}. Inhibition of mTOR restores the sensitivity of PTEN-deficient gliomas to EGFR inhibitors^{7,15,16}. Since 40% to 50% of glioblastomas lack PTEN expression, these findings were the rationale for the combined use of EGFR and mTOR inhibitors in human patients (see below). Furthermore, it became obvious that even in glioblastoma specimens with wild-

type PTEN expression, resistance to EGFR inhibitors may occur as a result of phosphorylation of PTEN at the conserved tyrosine residue Y240¹⁷.

One of the most comprehensive strategies aiming at identifying molecules and pathways that mediate resistance to EGFR inhibition has been the use of genome-wide small hairpin (sh)RNA screens. The application of such an approach revealed the dopamine receptor D2 (DRD2) signalling pathway as a novel therapeutic target. Combined inhibition of DRD2 signalling and EGFR inhibition resulted in synergistic anti-glioma activity in *in vitro* and *in vivo* models¹⁸.

Glioma cells can be characterized by intrinsic resistance to EGFR inhibitors or acquired mechanisms that allow them to escape from EGFR-targeted treatment. Acquired resistance to EGFR inhibition in EGFR-mutant glioma cells is conferred by an induction of platelet-derived growth factor receptor (PDGFR)- β expression. Consequently, the combined targeting of EGFR and PDGFR- β resulted in more potent anti-tumor activity in preclinical glioma models than either treatment alone¹⁹. Furthermore, expression of the *promyelocytic leukemia* (PML) gene in glioma cells prevents the induction of cell death in response to EGFR inhibition²⁰. Abrogation of PML expression by siRNA-mediated gene silencing or administration of the PML inhibitor arsenic trioxide restored the susceptibility of experimental gliomas *in vivo* to EGFR inhibition.

The fact that not all tumor cells share the same molecular make-up may also contribute to resistance to EGFR inhibition. In this regard, a population of cells within gliomas that exhibit stem cell-like properties has been described within the last years. These cells, also known as glioma-initiating cells (GIC), have been proposed as a major factor for the ultimately lethal course of the disease due to their contribution to the resistance of gliomas to various treatments. Preclinical data indicate that the resistance of GIC to EGFR inhibition is partially due to focal adhesion kinase (FAK)-mediated integrin β 1 signalling²¹. A similar study revealed that only co-treatment consisting of erlotinib and the hedgehog pathway inhibitor

cyclopamine had an effect on sphere initiation in glioblastoma stem-cell cultures²². These reports demonstrate that anti-EGFR strategies hold promise in targeting the stem cell population within glioblastomas. However, they also suggest that EGFR inhibition alone is insufficient and needs to be combined with the therapeutic targeting of at least one additional pathway. Further studies are required to define which combination may work best and which of the available combinations ultimately succeeds in human patients.

Clinical trials and combined treatment approaches in patients with malignant gliomas

Treatment of glioma patients with pharmacological EGFR inhibitors or blocking antibodies as single treatment has been largely futile. Several trials, using anti-EGFR approaches for gliomas with different WHO grades in the settings of newly diagnosed or recurrent tumors, have failed to show signs of activity. However, as outlined above, extensive preclinical work demonstrated that the disruption of converging signalling pathways may help to overcome resistance to EGFR inhibitors. Based on the increasing awareness that combinatorial targeting of EGFR and one or more additional molecule(s) may exert more robust anti-tumor activity, various trials were initiated using combinations of EGFR-targeting agents and additional drugs.

Clinical administration of TKI

Several preclinical reports suggest a sensitizing effect of EGFR inhibition to irradiation²³. Accordingly, clinical trials were designed to explore the combination of radiation therapy with EGFR inhibitors in patients with newly diagnosed glioblastoma. However, such trials failed to show benefit from addition of gefitinib or erlotinib to radiation therapy compared to historical controls. Notably, these trials completed enrolment before the introduction of

temozolomide to the standard of care for glioblastoma patients^{24,25}. Addition of erlotinib to temozolomide-based chemoradiation in patients with newly diagnosed glioblastoma resulted in prolonged overall survival compared to historical controls²⁶ but confirmation of this finding in a randomized trial is lacking and seems not to be further pursued. A phase I/II study explored the combination of the EGFR inhibitor lapatinib and the multikinase inhibitor pazopanib in patients with recurrent malignant glioma patients. Here, patients were stratified into 2 groups with either intact PTEN or EGFRvIII expression or without PTEN and EGFRvIII expression. However, the overall limited activity of this regimen did not differ among patients stratified by tumor EGFRvIII or PTEN status. A pharmacokinetic analysis demonstrated that only subtherapeutic levels of lapatinib were reached which may have precluded sufficient inhibition of EGFR signalling²⁷. The combined administration of the mechanistic target of rapamycin (mTOR) inhibitor, everolimus, and gefitinib did not achieve durable responses in patients with recurrent glioblastoma²⁸. Similarly, a phase I/II trial exploring the combination of erlotinib with the mTOR inhibitor temsirolimus in patients with recurrent malignant glioma failed to prove relevant anti-tumor activity. However, dose-limiting toxicity involving rash and mucositis was common²⁹. Low tumor levels of both drugs and the failure to prove target inhibition in the posttreatment tissue of several patients may partially explain the futility of this regimen. Compared to the “first-generation” EGFR inhibitors, second-generation, irreversible, TKI may exert more potent anti-glioma activity. However, one of these novel drugs, afatinib, did not show any signs of activity when used as single agent and did not improve the outcome of patients with recurrent glioblastoma in combination with temozolomide, likely because of negligible blood brain barrier penetration³⁰. Other compounds such as dacomitinib (PF-00299804) are currently being tested in clinical trials enrolling patients with recurrent glioblastoma (NCT01520870 and NCT01112527).

Anti-EGFR antibodies

The anti-EGFR antibody nimotuzumab was assessed in several clinical trials in patients with high-grade gliomas either alone or in combination with other treatment modalities and demonstrated only modest or no signs of activity^{31,32}. The lacking benefit of this regimen may be explained by the recent finding that treatment of glioma cells with an anti-EGFR antibody enhances DNA repair and thereby abrogates the effectiveness of DNA-damaging agents³³. Accordingly, the development of more elaborated strategies that combine irradiation and/or alkylating chemotherapy with anti-EGFR strategies is required. Novel anti-EGFR antibodies such as mAb806 target EGFRvIII and a subset of the overexpressed wild type EGFR but do not interact with wild-type EGFR expressed by normal cells. The administration of mAb806 has shown promising results in preclinical glioma models^{34,35}. However, data on its putative clinical activity are still lacking. Further strategies include the administration of antibody-drug conjugates (ADC) which comprise of an anti-EGFR antibody conjugated to potent cytotoxic drugs. ABT-414 is an ADC which mainly interacts with tumor cells expressing wild-type amplified EGFR or EGFRvIII. The activity of ABT-414 against glioblastoma is currently tested in clinical trials³⁶. In a similar approach, administration of a ¹²⁵I-labeled anti-EGFR antibody (¹²⁵I-MAb 425) in combination with radiation therapy did not result in better outcome compared to irradiation alone in patients with anaplastic glioma or glioblastoma³⁷.

Preclinical developments

Novel drug conjugates such as DAB389EGF, a fusion protein composed of diphtheria toxin linked to EGF have shown activity in experimental glioma models³⁸. Further approaches that have not yet reached the clinic include EGFR gene silencing by RNA interference or ribozyme-mediated cleavage of EGFR mRNA molecules^{39,40}. It needs to be awaited whether these techniques may become available for clinical testing in the future.

Resistance to therapeutic approaches specifically targeting EGFRvIII

EGFRvIII has been described as a mediator of glioma cell resistance to chemotherapeutic drugs *in vitro* through up-regulation of the anti-apoptotic protein Bcl-XL ⁴¹. However, the clinical impact of EGFRvIII expression on progression-free and overall survival has remained controversial. A report assessing tumor samples from 73 patients revealed an association of EGFRvIII expression with prolonged overall survival ⁴². These authors also reported that EGFRvIII-negative neurosphere cells are more resistant to temozolomide than EGFRvIII-positive cells suggesting that expression of EGFRvIII rather acts as a sensitizer to alkylating drugs. Notable, very high concentrations of TMZ were used for these *in vitro* studies which precludes translation into a clinical setting. Indeed, these findings are at odds with other reports. A study by Shinojima and colleagues revealed an association between EGFRvIII expression and poor overall survival in glioblastoma patients ⁴³. Ultimately, a comprehensive analysis of more than 180 glioblastoma patients demonstrated that the clinical course of EGFRvIII-expressing glioblastomas is not significantly different from that of patients harbouring EGFRvIII-negative tumors. However, long-term survival, defined by overall survival of more than 3 years, was virtually absent in patients with EGFRvIII-positive glioblastoma ⁴⁴.

Expression of EGFRvIII also defines a subgroup of tumor cells within a glioblastoma with stem cell characteristics ⁴⁵. It was reported that EGFRvIII is coexpressed with the putative stem cell marker CD133 and that these cells are characterized by the highest degree of self-renewal as well as a pronounced tumorigenicity *in vivo*. On the cellular level, there is a close oncogenic signalling relationship between wild-type EGFR and EGFRvIII which drives glioblastoma progression ⁴⁶. Furthermore, a minority of cells within a glioblastoma expressing the EGFRvIII mutant may be sufficient to promote tumor growth by inducing the expression

of several cytokines such as interleukin (IL)-6 and leukemia inhibitory factor (LIF). Subsequently, these cytokines act in a paracrine manner on EGFRvIII-negative cells in the neighbourhood by accelerating their proliferation⁴⁷. Accordingly, the specific targeting of EGFRvIII may have an impact on tumor growth beyond the population of EGFRvIII-positive cells. Proteomic analyses revealed that the expression of EGFRvIII results in the activation of different downstream pathways compared to gliomas that are EGFRvIII-deficient⁴⁸. In this regard, preclinical data suggest a synergistic activity when EGFRvIII inhibition is combined with targeting of an additional pathway such as c-MET signalling or the urokinase-type plasminogen activator (uPAR) receptor pathway^{49,50}. It was also reported that resistance to anti-EGFR strategies is associated with increased expression of EGFRvIII and an activation of the PI3K pathway. The latter is accompanied by an induction of the expression of the regulatory 110-kDa delta subunit of PI3K (p110 δ). Preclinical findings suggest that insulin-like growth factor receptor (IGFR)-I signaling via PI3K and the presence of major vault proteins (MVP) which stabilize EGFR/PI3K signalling also contribute to resistance to anti-EGFR therapy in glioma cells^{51,52}. Silencing of EGFRvIII resulted in a sensitization to the EGFR inhibitor erlotinib. Similarly, targeting PI3K or the p110 δ subunit also restored erlotinib sensitivity⁵³. Considering these findings, the selective interfering with EGFRvIII signalling may help to overcome the treatment resistance of glioblastomas.

Immunotherapy: escape from vaccination against EGFRvIII

Since EGFRvIII is exclusively expressed on tumor cells, it represents an appealing target for therapeutic interventions. In contrast to wild-type EGFR which has been used as a point of attack for pharmacological inhibitors or antibodies, EGFRvIII has gained additional interest as target structure for active immunotherapy, that is, vaccination which aims at overcoming the immune evasion of glioma cells⁵⁴. Currently, a peptide-based vaccine (CDX-110, also

known as rindopepimut) is in late stage clinical development and assessed in combination with standard temozolomide-based chemoradiation in patients with newly diagnosed glioblastoma with proven expression of EGFRvIII (ACT IV, NCT01480479) as well as in a phase II study in patients with relapsed EGFRvIII-positive glioblastoma (ReACT, NCT01498328). This vaccine has already shown promising results in smaller trials and prolonged survival compared to matched historical controls. However, it was also reported that the expression of EGFRvIII is lost upon vaccination with rindopepimut when tissue specimens from recurrent tumors were compared to the tumor tissue at initial diagnosis⁵⁵. On the one hand, this may indicate the activity of the vaccine and the removal of tumor cells expressing the target antigen by the immune system. On the other hand, it also suggests immune evasion by the tumor due to loss of the target structure. This process, also known as “cancer immunoediting” precludes durable immune responses against glioma cells unless further tumor antigens are recognized by the effector mechanisms of the immune system⁵⁶. Currently, various strategies aiming at boosting the immune system against cancer are investigated in clinical trials. Immune checkpoint inhibitors targeting programmed cell death (PD)-1 or cytotoxic T lymphocyte antigen (CTLA)-4 as well as drugs which may help to overcome the immunosuppressive environment surrounding glioma, e.g. by suppressing transforming growth factor (TGF)- β signalling, may allow for more powerful immune responses against gliomas. However, whether the combination of any of these novel approaches with a vaccine against EGFRvIII prevents escape from vaccination alone and results in sustained clinical benefit, must be determined within clinical trials.

Outlook

TCGA has reconfirmed the EGFR gene as a principal target of mutation in glioblastoma, but also illustrated the variability of alterations. The history of negative trials with EGFR-targeted

agents teaches us that any future effort at exploiting this target for therapy must be based on a molecularly defined patient enrichment including at least EGFR status, but potentially also changes in associated pathways (Fig. 1). Advances with the use of anti-EGFR treatments will require stratification based on the presence of EGFR overexpression or amplification as well as the presence of the EGFRvIII mutation. Patients with tumors harbouring any of these alterations may be most likely to benefit from EGFR-targeted therapies. While the fate of the immunotherapeutic efforts targeting EGFRvIII will depend on the outcome of the currently ongoing trials, the availability of antibody-drug conjugates as well as the selection of drug combinations selected upon individual tumor tissue examination may pave the road for more successful therapies.

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Figure legend

Figure 1. Treatment approach to glioblastoma based on EGFR-stratification.

Abbreviations: CAR, chimeric antigen receptor; EGFR, epidermal growth factor receptor; EGFRvIII, EGFR variant III; mTOR, mechanistic target of rapamycin; PDGFR- β , platelet-derived growth factor receptor β . ClinicalTrials.gov identifier: ACT IV (NCT01480479), ReACT (NCT01498328)